AD_____

Award Number: W81XWH-04-1-0251

TITLE: The Role of CXCR4 and Arrestins in Breast Cancer Signaling and Apoptosis

PRINCIPAL INVESTIGATOR: Brant M. Wagener

CONTRACTING ORGANIZATION: Regents of the University of New Mexico
Albuquerque, NM 87131

REPORT DATE: February 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-02-2006	Annual	1 Feb 2005 - 31 Jan 2006

4. TITLE AND SUBTITLE
The Role of CXCR4 and Arrestins in Breast Cancer Signaling and Apoptosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0251

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Brant M. Wagener

E-Mail: bwagener@salud.unm.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Regents of the University of New Mexico
Albuquerque, NM 87131

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: We use the N-formyl peptide receptor (FPR) as a model for G protein-coupled receptor (GPCR) activity in the absence of arrestins. GPCRs are beginning to take center stage in understanding cancer metastasis, specifically breast cancer metastasis. Also, GPCRs are integral to cell migration, another important feature of metastasis. Arrestin is a major player in GPCR trafficking and signaling both at the cell surface and post-endocytically. Therefore, understanding GPCR function in the absence of arrestins may lead to novel chemotherapeutics for the treatment of breast cancer. We use the FPR as a model for the system we proposed in our original submission as it is a member of the same receptor family, reagents are available that make the research methods easier and the same fundamental questions can be answered about G protein-coupled receptors (GPCR) and arrestins in breast cancer. We have previously found mutants of arrestin the do not rescue FPR-mediated apoptosis. Also, these mutants show trafficking defects and may be related to AP-2, a regulator of cellular trafficking. In this report, we outline further experiments performed to elucidate to role of AP-2 in FPR/arrestin function to better understand the mechanism of FPR-mediated apoptosis. In addition, we have found other mutants of arrestin (some of which have deficient interaction with Src kinase) that also do not rescue FPR-mediated apoptosis. We show data that helps us understand the role of Src kinase in this process as well. Finally, data from all three tasks lead to the hypothesis that there may be multiple, independent steps gone awry in FPR-mediated apoptosis in the absence of arrestins. These steps seem likely to be regulated by interaction and signaling form arrestins, Src kinase AND AP-2, perhaps in combination. Finally, data from this grant may lead to further funding with the UNM Screening Center for small molecules that interrupt the interaction of these proteins which would be the first step towards finding a novel chemotherapeutic with which to treat metastatic breast cancer.

15. SUBJECT TERMS
GPCR, Apoptosis, Arrestin

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	29	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION

In the previous annual report submitted, we had changed the focus of the proposal from CXCR4 to the N-formyl peptide receptor (FPR). This was due to the fact that published data demonstrated that CXCR4 stimulation did not cause apoptosis in the absence of arrestins, but the FPR did (*1*). Also, the FPR works well as a model for the system we proposed in our original submission as it is a member of the same receptor family, reagents are available that make the research methods easier and the same fundamental questions can be answered about G protein-coupled receptors (GPCR) and arrestins in breast cancer. Finally, in the last proposal, we provided an update of Task One (the only one worked on) in which we had designed arrestin mutants and assayed them for their effects in FPR-mediated apoptosis, FPR internalization and trafficking with the FPR and Rab11. It was demonstrated that there was a correlation between FPR-mediated apoptosis and Rab11 trafficking, but none with FPR internalization. Also, it was realized that both arrestin mutations that do not rescue FPR-mediated apoptosis have known or suspected interactions with AP-2. This report details further experiments elucidating this interaction and work done in Tasks Two and Three. Some details in the Statement of Work have changed and a revised statement was submitted in February 2006.

BODY

Task One. From the previous report, we found two arrestin mutants that did not rescue FPR-mediated apoptosis: arr2-F391A and -4A. Arr2-F391A has been previously described to be deficient in binding to AP-2 (*2*). Arr2-4A consists of arr2-397K, -399M and -400K all changed to alanine. These three individual mutations also showed decreased binding to AP-2 although nothing is known about them in combination (*2*). To help us understand arrestin mutant interaction with AP-2 as a consequence of FPR activation, arrestin knockout mouse embryonic fibroblasts stably transduced with the FPR (KOFPR) were transiently transfected with RFP-fused arrestin constructs and a GFP-fused member of the AP-2 tetramer. Cells were then plated to glass coverslips, stimulated with 10nM 633-6pep for various times, fixed and mounted. Cells were imaged using confocal fluorescence microscopy. In Figure 1, it can be seen that cellular trafficking of the wild-type and mutant arrestins and the FPR are consistent with Rab 11 colocalization from the previous annual report (mutant arrestins and empty vector accumulate in a perinuclear location and wild-type arrestin is present there, but also in other areas of the cytoplasm). Also, at zero time points for all arrestins, AP-2 is visualized at the plasma membrane. Upon FPR activation, AP-2 appears not to colocalize with FPR in the absence of arrestins or in the presence of arr2-F391A, consistent with previous binding data. AP-2 does colocalize with arr2-WT (as expected) and arr2-4A which is an unexpected result. It also appears from the 60 minute time points that **more** AP-2 may be present with arr2-4A than with arr2-WT. Also, Figure 2 indicates that at earlier time points AP-2 colocalizes with arr2-4A and –WT, but apparently never with empty vector or arr2-F391A.

It is entirely possible that AP-2 may be colocalizing with the FPR and arr2-WT or -4A, but not physically binding either arrestin or that AP-2 does not colocalize with the FPR and arr2-F391A but does bind to it and release somewhere on its path to the perinuclear region. To answer this question we performed co-immunoprecipitations of arrestins and AP-2 at various times of FPR activation. KOFPRs were transiently transfected with flag-tagged arr2-WT, -4A, or –F391A or empty pcDNA3 vector and seeded for confluence in 10cm dishes. Cells were serum-starved for 30 minutes, stimulated with 10nM fMLF for varying times and lysed. Lysates were centrifuged, an aliquot was kept to measure proteins in cellular lysates and the remainder was immunoprecpitated using M2 Anti-FLAG antibody on sepharose beads overnight at 4°C. Samples were resolved by SDS-PAGE, transferred to PVDF and blotted with appropriate primary and secondary antibodies according to standard protocols. As can be seen in Figure 3, all samples show good endogenous levels of β-adaptin (subunit of AP-2 that binds arrestin) and flag-tagged arr2-WT, -4A and –F391A were transiently expressed at adequate levels. In the immunoprecipitations, relatively equal levels of flag-tagged arrestins were collected. Finally, in the IPs, wild-type arrestin increasingly binds β-adaptin to a maximal amount at 30 minutes and disappears at 60 minutes. Arr2-F391A doesn't appear to bind β-adaptin at all in response to FPR activation and arr2-4A seems to bind more strongly than arr2-WT and does not release at 60 minutes.

AP-1 is a closely related cousin of AP-2 showing significant homology (83%) and almost exact sequence homology to AP-2 in the subunit region that binds arrestin (*3*). In addition, AP-1 has been demonstrated to be located in recycling endosomes and necessary for the recycling of mannose 6-phosphate receptors (*4*). Based on this data, we hypothesized that arrestin binding to AP-1 might be needed for proper trafficking of the FPR back to the cell surface. To help us answer this question we transiently transfected KOFPRs with RFP-fused arrestins (or empty vector) and a GFP-fused subunit of the AP-1 tetramer. Cells were then plated to glass coverslips, stimulated with 10nM 633-6pep, fixed and mounted. Slides were imaged using confocal fluorescence microscopy. In all unstimulated samples, AP-1 can be seen in a perinuclear region that may be the same as the Rab11 compartment (Figure 4). After stimulation of the FPR, the receptor and arrestins (or empty vector) behave as seen in the previous report and Figures 1 and 2. For empty RFP vector and arr2-4A and –F391A, receptor and arrestins seem to accumulate with the AP-1 compartment. However, arr2-WT passes through this compartment and receptor, arrestin and AP-1 can be seen in vesicles that appear to be trafficking normally. Based on this data, it appears that there may be an interaction between arrestin, AP-2 and AP-1 that is necessary for normal FPR trafficking (discussed below).

We have numerous microscopic data demonstrating that arr2-4A and -F391A do not allow FPR trafficking to the cell surface. In addition, we have previously shown that in the absence of arrestins, the FPR does not recycle (*5*). To provide quantitative data demonstrating this phenomenon, we transiently transfected KOFPRs with EGFP or GFP-fused arr2-WT, -4A and –F391A. Cells were harvested, resuspended in cold serum-free medium (SFM) and one-third

were aliquoted and labeled with 633-6pep (total cell surface receptor). The remainder were stimulated with 1μM fMLF for one hour at 37°C. Cells were then washed extensively with cold SFM to remove excess unlabelled ligand and split into two aliquots. The first aliquot was labeled with 633-6pep (cell surface receptor after internalization) and the second aliquot was resuspended in pre-warmed SFM and allowed to recycle for 30 minutes at 37°C. The final aliquot (cell surface receptor recycled) is then labeled with 633-6pep and samples are analyzed by flow cytometry. Data are organized by plotting the ratio of receptor recycled/receptor internalized. As can be seen in Figure 5, cells transfected with arr2-WT traffic similarly to previously published data (*5*) (~20%) whereas EGFP transfected cells do not recycle at all. Both arr2-4A and –F391A do not rescue the recycling of the FPR when transfected into KOFPRs suggesting that altered AP-2 interaction can affect the trafficking of this GPCR.

To summarize the results of Task One, we have created a model of FPR trafficking and the proteins that interact with it found in Figure 6. After the receptor binds ligand, it internalizes and binds arrestin. At some point, it binds to AP-2 and moves to the perinuclear region where AP-1 already resides. Next, arrestin must release AP-2 and bind to AP-1 to allow it to move away from the perinuclear region. Finally, the receptor/arrestin/AP-1 complex dissociates and the receptor returns to the cell surface to begin the cycle again. This model is supported by a few facts. First, arrestin is necessary for recycling (Figure 6 and *). Second, one arrestin mutant does not bind AP-2 and the other binds more tightly that arr2-WT suggesting that binding and release is necessary for recycling. Finally, only arr2-WT is seen with AP-1 somewhere other than the perinuclear region suggesting it aids the receptor's return to the cell surface. These data are currently being formed into a publication (listed below) and should be submitted within one month.

Task Two. In order to understand the role of signaling molecules in FPR-mediated apoptosis in the absence of arrestin, we built an arrestin mutant (P91G/P121E or arr2-PP) that has been previously demonstrated to be deficient of binding Src kinase and inhibits the internalization of the β2-adrenergic receptor (β2-AR) (*6*). KOFPRs were transiently transfected with EGFP, arr2-WT-GFP and arr2-PP-GFP, plated to coverslips, serum-starved for 30 minutes and stimulated with 10nM 633-6pep or given SFM alone for 5 hours. Cells were then stained with propidium iodide (PI), washed with PBS, fixed and mounted. Cells were viewed by fluorescence microscopy and 100-300 GFP expressing cells were counted. Of these, cells were noted to be PI positive or negative. Figure 7 shows this data expressed as PI positive/GFP expressing cell. Only expression of arr2-WT rescues FPR-mediated apoptosis whereas EGFP vector and arr2-PP do not.

While this mutant has been demonstrated to be Src kinase-binding deficient, there is some question as to whether Src kinase remains in an active state. It may be that uncontrolled Src kinase activity is contributing to the apoptotic phenotype. To help answer this question, we ran our apoptosis assay (as described in the paragraph above) with EGFP, arr2-WT-GFP and arr2-PP-GFP, but every condition is stimulated in the presence of either DMSO or the Src

family kinase inhibitor, PP2. Cells were pretreated with both for 30 minutes and DMSO and inhibitor were present throughout the entire stimulation. As seen in Figure 8, PP2 stops FPR-mediated apoptosis allowed by EGFP and arr2-PP indicating that although Src kinase may not be binding this arrestin mutant, Src activity is still playing a role in FPR-mediated apoptosis.

To understand if arr2-PP traffics in similar patterns to other arrestin mutants that do not rescue FPR-mediated apoptosis, we transiently transfected KOFPRs with RFP-fused arr2-WT and –PP and GFP-fused Rab11, or subunits of AP-2 and AP-1. Cells were then plated to glass coverslips, treated with 10 nM 633-6pep, fixed and mounted as described above. Coverslips were imaged by confocal fluorescence microscopy. Figures 9-11 show the result of these experiments. In Rab11 experiments (Figure 9), empty vector and arr2-WT behave similarly to previous published results and previous reports (*5*) where receptor accumulates in the absence of arrestin and traffics normally in the presence of wild-type arrestin. However, similar to other arrestin mutants (described above), arr2-PP and FPR accumulate and are presumably not trafficking normally. In AP-2 experiments (Figure 10), empty vector and arr2-WT behave as they normally do (see Figure 1) and arr2-PP appears to colocalize with AP-2. Whether this indicates that direct binding is taking place is unclear. Src activity is known to be necessary for phosphorylation of tyrosines on AP-2 and release from arrestin (*7*), but since arr2-PP does not bind Src kinase, this activity may not be taking place. Finally, in AP-1 experiments (Figure 11), empty vector and arr2-WT are behaving as previously described (Figure 3). Arr2-PP and AP-1 do not colocalize outside of the perinuclear region (as arr2-WT does), once again indicating that in the presence of arr2-PP, the FPR does not traffic properly and is likely not recycling to the cell surface.

Finally, as mentioned, reports demonstrate that Src kinase activity is integral to arrestin-AP-2 interaction (*7*). We believed that if Src kinase binding to arrestin or its activity is causing apoptosis and Task One demonstrates that these phenotypes are linked, how would Src kinase inhibitors affect receptor/arrestin trafficking? To address this question, we transiently transfected KOFPRs with Rab11-GFP and RFP-fused arrestins. Microscopic analysis was run as described above, but with pretreatment of cells with DMSO or Src family kinase inhibitor, PP2, and treatment during stimulation. Results of this experiment can be seen in Figure 12. All DMSO treated cells show the same phenotypes of trafficking normally or accumulation as seen in Figure 9. However, cells treated with PP2 show interesting phenotypes. First, PP2 treatment does not rescue accumulation seen with empty vector or arr2-PP. However, PP2 treatment does appear to stop normal trafficking of arr2-WT and force accumulation. Also, in Figure 8, PP2 treatment of arr2-WT transfected cells did not cause apoptosis.

The data attained to date in this task leads us to believe that there must be two independent Src kinase events taking place upon FPR activation—one that controls the trafficking of the receptor and one that leads to apoptosis. In order to better understand these phenomena, we will next stain KOFPRs with Src kinase and phospho-Src kinase antibodies under varying conditions and view the location of Src kinase by confocal fluorescence microscopy. This will help us

understand the spatial activity of Src and the mechanism of its role in FPR-mediated apoptosis.

Task Three. As a solution to part of the questions raised in Task Two, we designed three mutants that have mutations in SH3 binding domains of arrestin. The mutant in Task Two had two prolines mutated—one proline in two different SH3 motifs. The mutants we designed (Figure 13) have both prolines in each of the individual SH3 motifs changed to alanine. These mutants are designated arr2-M1, -M2 and –M3. In this way we can understand if one motif is responsible for varying arrestin-Src kinase functions.

To understand the role of these mutants in FPR-mediated apoptosis, KOFPRs were transiently transfected with EGFP and GFP-fused arr2-WT, -M1, -M2 and –M3 and assayed for apoptosis as described in Task Two. As can be seen in Figure 14, EGFP and arr2-WT-GFP controls worked as described above. Arr2-M1 and –M3 rescued FPR-mediated apoptosis whereas arr2-M2 did not. This indicates that the second SH3-binding motif appears to be responsible for FPR-mediated apoptosis.

Previous reports have demonstrated that when the FPR cannot internalize, the cell does not undergo FPR-mediated apoptosis (*1*). To understand whether these mutants inhibit the internalization of the FPR, we assayed the internalization of the FPR in the presence of GFP-fused arrestin-2 mutants generated above. KOFPRs transiently transfected with GFP-fused arrestin-2 mutants were assayed for FPR internalization. The FPR internalizes in the presence of arrestin-2 mutants as well or better than (EGFP) empty vector (Figure 15). At this time, we conclude that any arrestin-2 mutant that will inhibit FPR-mediated apoptosis is doing so for some other reason than preventing the FPR from leaving the cell surface. Untransfected KOFPRs and WTFPRs were also assayed and consistent with previous published results.

To understand if arr2-M1, -M2 and –M3 traffic in similar patterns to other arrestin mutants that do or do not rescue FPR-mediated apoptosis, we transiently transfected KOFPRs with RFP-fused arr2-WT, -M1, -M2 or –M3 and GFP-fused Rab11, or subunits of AP-2 and AP-1. Cells were then plated to glass coverslips, treated with 10 nM 633-6pep, fixed and mounted as described above. Coverslips were imaged by confocal fluorescence microscopy. Figures 16-18 show the result of these experiments. In Rab11 experiments (Figure 16), empty vector and arr2-WT behave similarly to previous published results and previous reports (*5*) where receptor accumulates in the absence of arrestin and traffics normally in the presence of wild-type arrestin. However, arr2-M1, -M2 and –M3 traffic normally similar to arr2-WT. This is interesting as arr2-M2 traffics normally as opposed to accumulating like every other arrestin mutant that does not rescue FPR-mediated apoptosis. In AP-2 experiments (Figure 17), empty vector and arr2-WT behave as they normally do (see Figure 1) and arr2-M1, -M2 and –M3 appear to colocalize with AP-2. Whether this indicates that direct binding is taking place is unclear. Finally, in AP-1 experiments (Figure 18), empty vector and arr2-WT are behaving as previously described (Figure 3). Arr2-M1, -M2 and –M3 colocalize outside of the perinuclear region with AP-1 (as arr2-WT does),

indicating that in the presence of arr2-M1, -M2 and –M3, the FPR traffics properly and is likely recycling to the cell surface.

These results are interesting as we have found an SH3-binding mutation of arrestin that does not rescue FPR-mediated apoptosis, but does allow the FPR to traffic normally. This further substantiates our findings in Task Two that Src kinase has two independent functions in FPR signaling and trafficking. The spatial signaling of Src kinase will be important to know as in Task Two as well as the possibility of building more arrestin mutants may help us understand this mechanism.

KEY RESEARCH ACCOMPLISHMENTS

- We have demonstrated that AP-2 has differential interaction with arrestin mutants by both fluorescence microscopy and co-immunoprecipitation.
- We have demonstrated that AP-1 is likely to be involved in FPR/arrestin post-endocytic trafficking.
- We have found that both arrestin mutants involved in Task One show a defect in FPR recycling.
- We have constructed a model of FPR trafficking based on evidence attained in Task One.
- We have shown that a mutant of arrestin deficient in Src binding (PP) does not rescue FPR-mediated apoptosis and this effect is sensitive to Src family kinase inhibitor.
- We have shown that PP mutant trafficking with Rab11 is similar to when no arrestin is present.
- PP mutant appears to bind to AP-2 by microscopy.
- PP mutant trafficking with AP-1 is similar to mutants in Task One.
- Src family kinase inhibitor does not change trafficking pattern of PP mutant/FPR or FPR in the absence of arrestin, but does seem to inhibit trafficking of wild-type arrestin.
- Mutants of arrestin with differing mutations in SH3-binding motifs show varying patterns of FPR-mediated apoptosis.
- These SH3 mutants effect on apoptosis is independent of their effect on internalization.
- All SH3 mutants appear to traffic normally with Rab11, AP-2 and AP-1 despite their differing effects on FPR-mediated apoptosis.

REPORTABLE OUTCOMES

- Presented poster on research from Task One at Era of Hope Conference in Philadelphia, PA—June 2005. “Arrestin Domains that Regualte N-Formyl Peptide Receptor Trafficking and Signaling.”
- Awarded Biomedical Sciences Graduate Program Travel Award.
- Manuscript in Preparation – “Arrestin-2 interaction with adaptor proteins regulates *N*-formyl peptide receptor post-endocytic trafficking.”

CONCLUSION

These results demonstrate that there are specific regions of arrestin responsible for controlling FPR-mediated apoptosis. In addition, trafficking of the receptor/arrestin complex is important to the initiation of apoptosis is regulated by interactions between AP-2, AP-1 and Src kinase. Future work will address the signaling differences in arrestin mutants that differentially regulate apoptosis and FPR trafficking as well as temporal and spatial signaling interactions with Src kinase.

These findings are important as GPCRs have been shown to be overexpressed in cancer cells (most notably breast cancer) and more are likely to be found. As all GPCRs interact with arrestin, understanding GPCR/arrestin interaction is crucial to understanding the role of GPCRs in metastatic breast cancer cells. GPCRs are not only involved in metastasis, but migration of metastatic cells is aided by these receptors. Understanding the role of arrestins with GPCRs could lead to novel chemotherapeutic therapy for breast cancer metastasis that may reduce metastasis by inhibiting migration or inducing apoptosis in breast cancer cells overexpressing GPCRs.

REFERENCES

1. C. M. Revankar, C. M. Vines, D. F. Cimino, E. R. Prossnitz, *J Biol Chem* **279**, 24578-84 (Jun 4, 2004).
2. Y. M. Kim, J. L. Benovic, *J Biol Chem* **277**, 30760-8 (Aug 23, 2002).
3. R. Lundmark, S. Carlsson, *Biochemical Journal* **362**, 597-607 (2002).
4. C. Meyer *et al.*, *Embo J* **19**, 2193-203 (May 15, 2000).
5. C. M. Vines *et al.*, *J Biol Chem* **278**, 41581-4 (Oct 24, 2003).
6. L. M. Luttrell *et al.*, *Science* **283**, 655-61 (Jan 29, 1999).
7. D. Fessart, M. Simaan, S. A. Laporte, *Mol Endocrinol* **19**, 491-503 (Feb, 2005).

APPENDIX

ARRESTIN DOMAINS THAT REGULATE N-FORMYL PEPTIDE RECEPTOR TRAFFICKING AND SIGNALING

Brant M. Wagener And Eric R. Prossnitz
University of New Mexico School of Medicine
bwagener@salud.unm.edu

Recent reports by our laboratory have demonstrated that arrestins are required for proper trafficking of the N-formyl peptide receptor (FPR). We used mouse embryonic fibroblasts (MEF) that are deficient in arrestin-2, -3 or both (2KO), but stably expressing the FPR, to study the signaling and trafficking of the FPR in the absence of one or more arrestins. We find that after simulation with formyl-Met-Leu-Phe (fMLF), recycling of the FPR is inhibited in the absence of both arrestins and that the internalized receptor is colocalized with the Rab11 GTPase in a perinuclear location.

A second study demonstrates 2KOs stably expressing the FPR undergo apoptosis when stimulated with fMLF. This phenotype was rescued by transfection with arrestin-2 WT and/or arrestin-3 WT cDNAs, but not by arrestin mutant cDNAs comprising important structural regions of the protein. In addition, FPR mutants that are incapable of either internalizing or signaling also do not initiate apoptosis when the receptor binds ligand.

The results of these two reports indicate that some trafficking and signaling defects of the FPR are arrestin-dependent. In addition, the mutants used in the second report lead us to believe that the region of arrestin responsible for these phenotypes lies in the tail of the protein (amino acids 383-419). We have constructed eleven mutants of arrestin-2. These arrestin-2 mutants include the mutation of the clathrin binding site, the AP-2 binding site and the serine phosphorylated by ERK 1/2. We have used these mutants to identify the region of arrestin-2 that regulates FPR trafficking and arrestin-dependent apoptosis. These studies have not only indicated the region of arrestin-2 that may be responsible, but has helped us to elucidate the signaling and trafficking mechanisms that may be responsible.

These studies will help us to better understand the role that arrestin and other molecules play in non-classical GPCR trafficking and signaling. In addition, these studies can be used as a model to better understand GPCR signaling and trafficking in a variety of disease states including breast cancer. Finally, understanding the role of arrestins in GPCR trafficking and signaling will allow the design of novel chemotherapeutics to target breast cancer cells abnormally expressing GPCRs including, but not limited to, CXCR4 and IL-8R.

The U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0251 supported this work.

SUPPORTING DATA

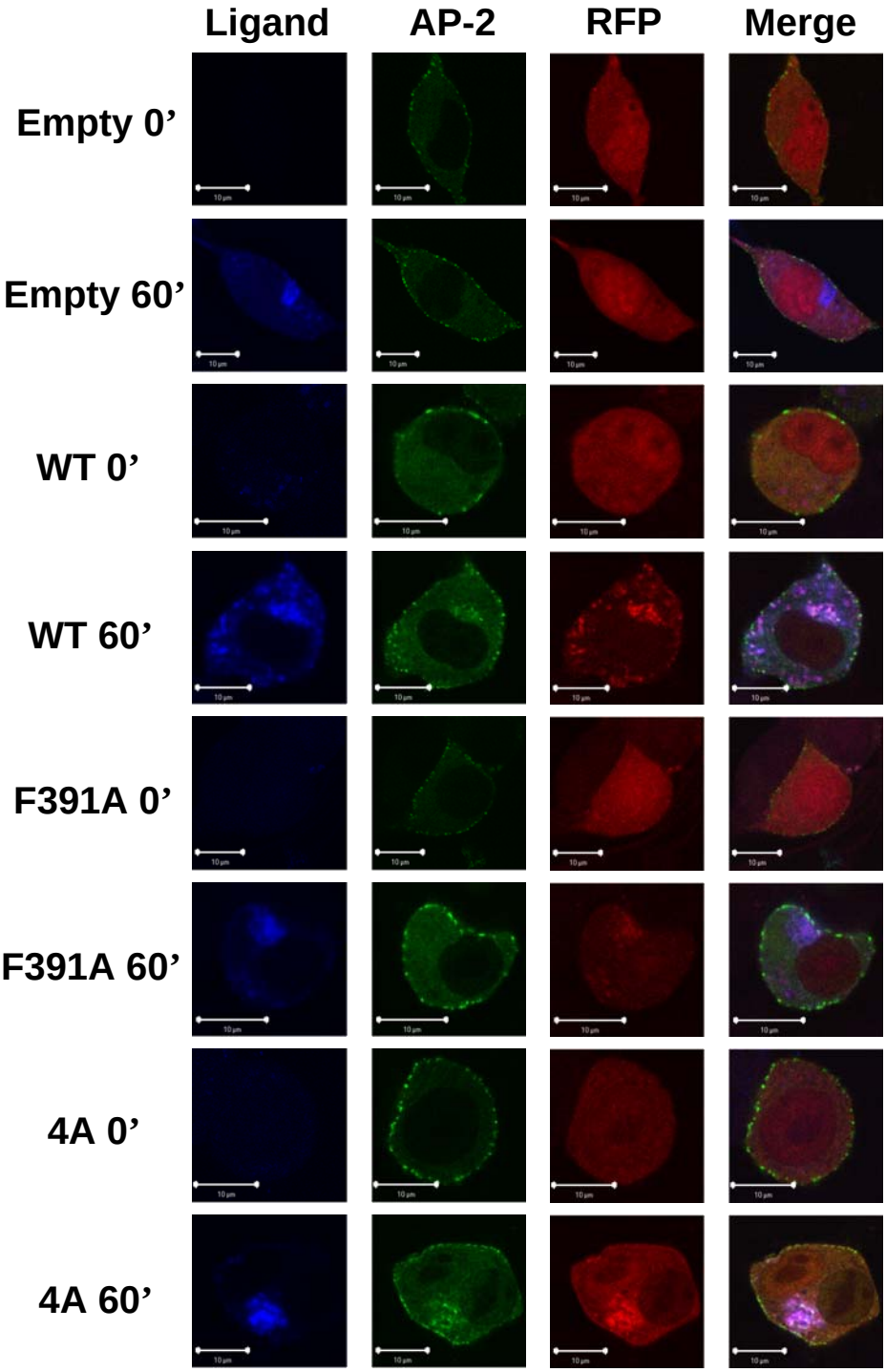

Figure 1. Arrestin mutants show differential binding to AP-2. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 63-3pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of three independent experiments.

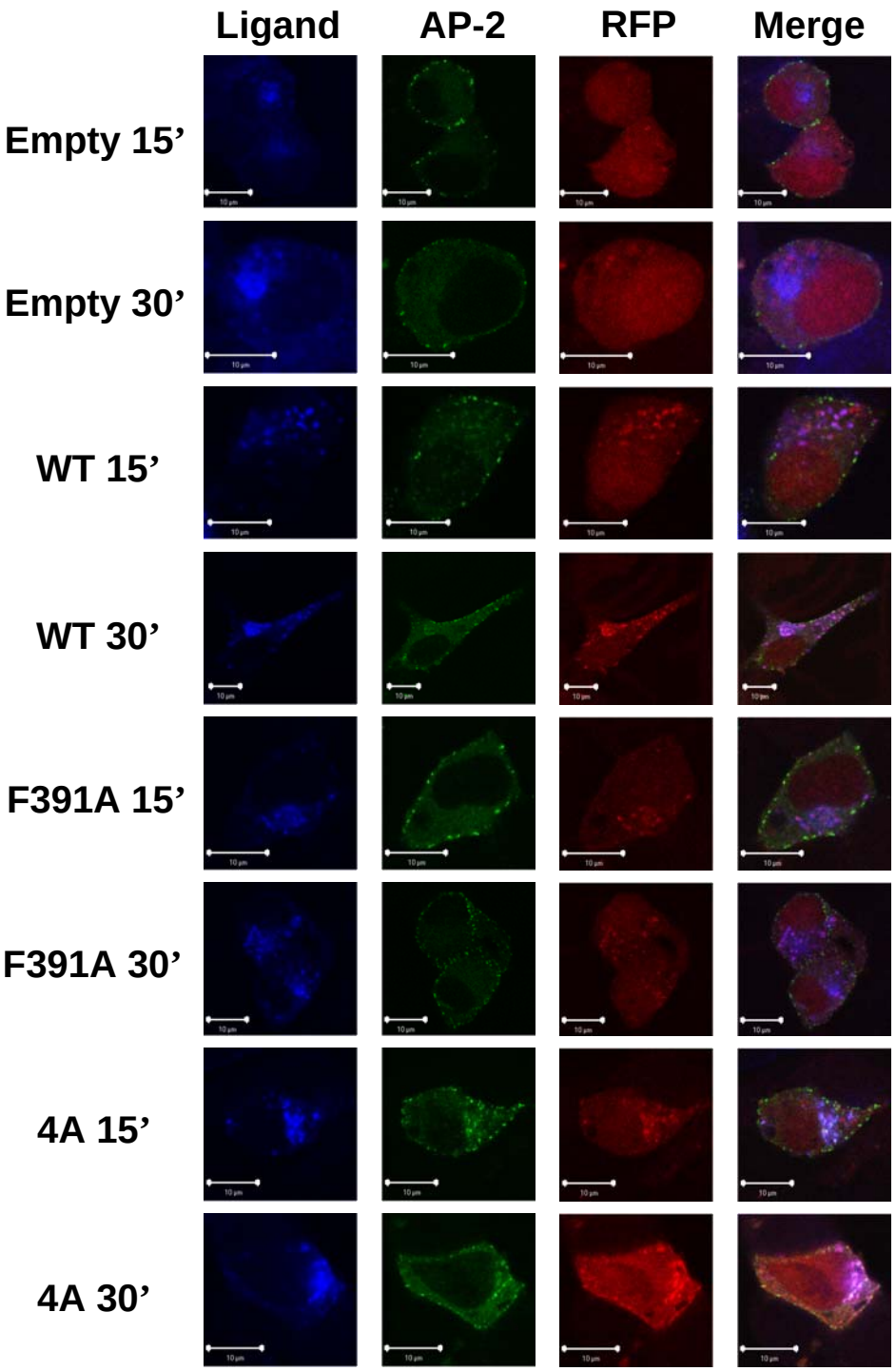

Figure 2. Arrestin mutants show differential binding to AP-2. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 63-3pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of three independent experiments.

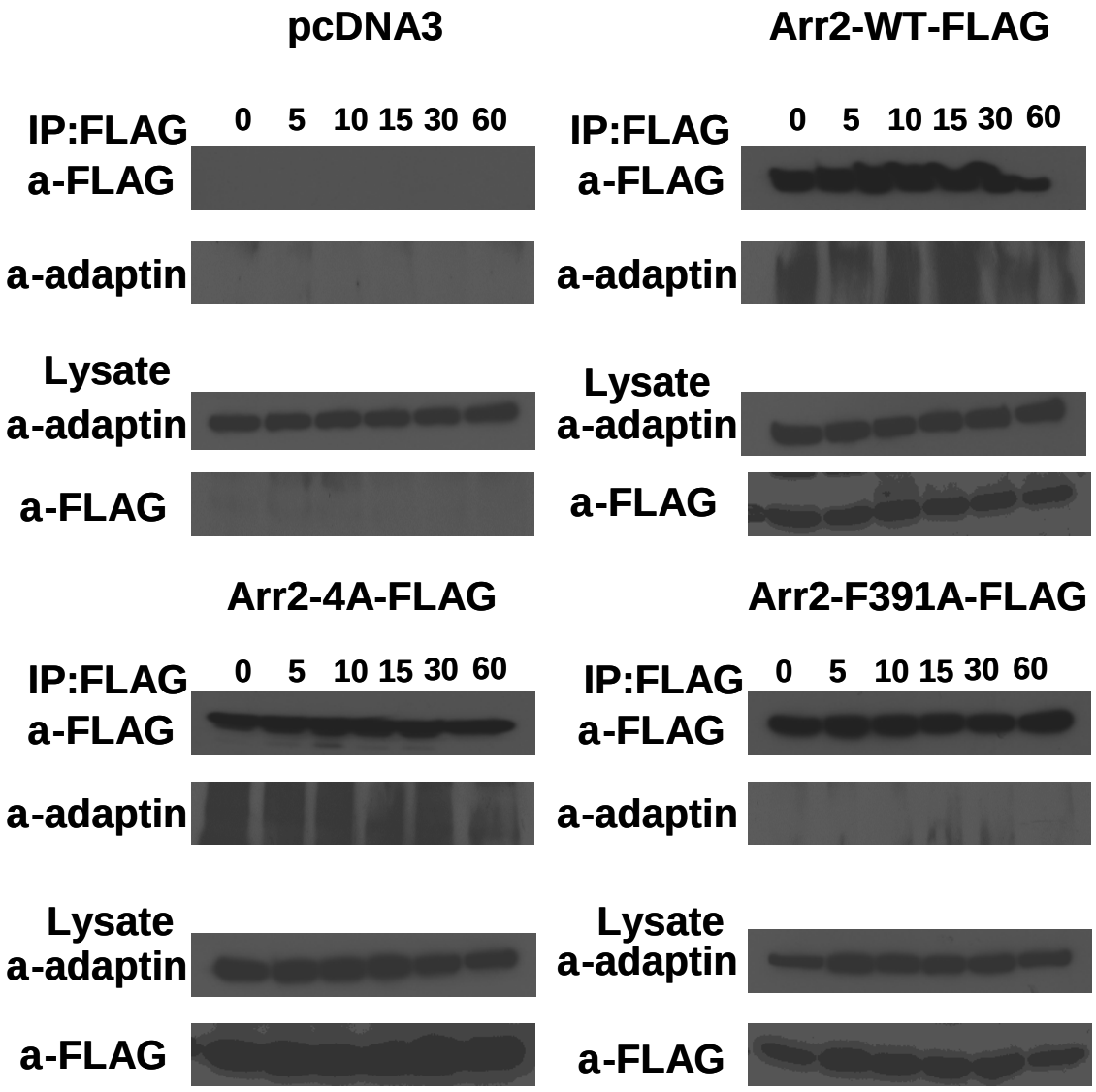

Figure 3. Arrestin interaction with ß-adaptin subunit of AP-2. Transiently transfected KOFPRs were stimulated with 10nM fMLF for varying times and lysed. Lysates were then Immunoprecipitated with M2-Anti-FLAG antibody, resolved by SDS-PAGE, transferred to PVDF and blotted as described in the figure. Representative blots are shown and are Indicative of two independent experiments.

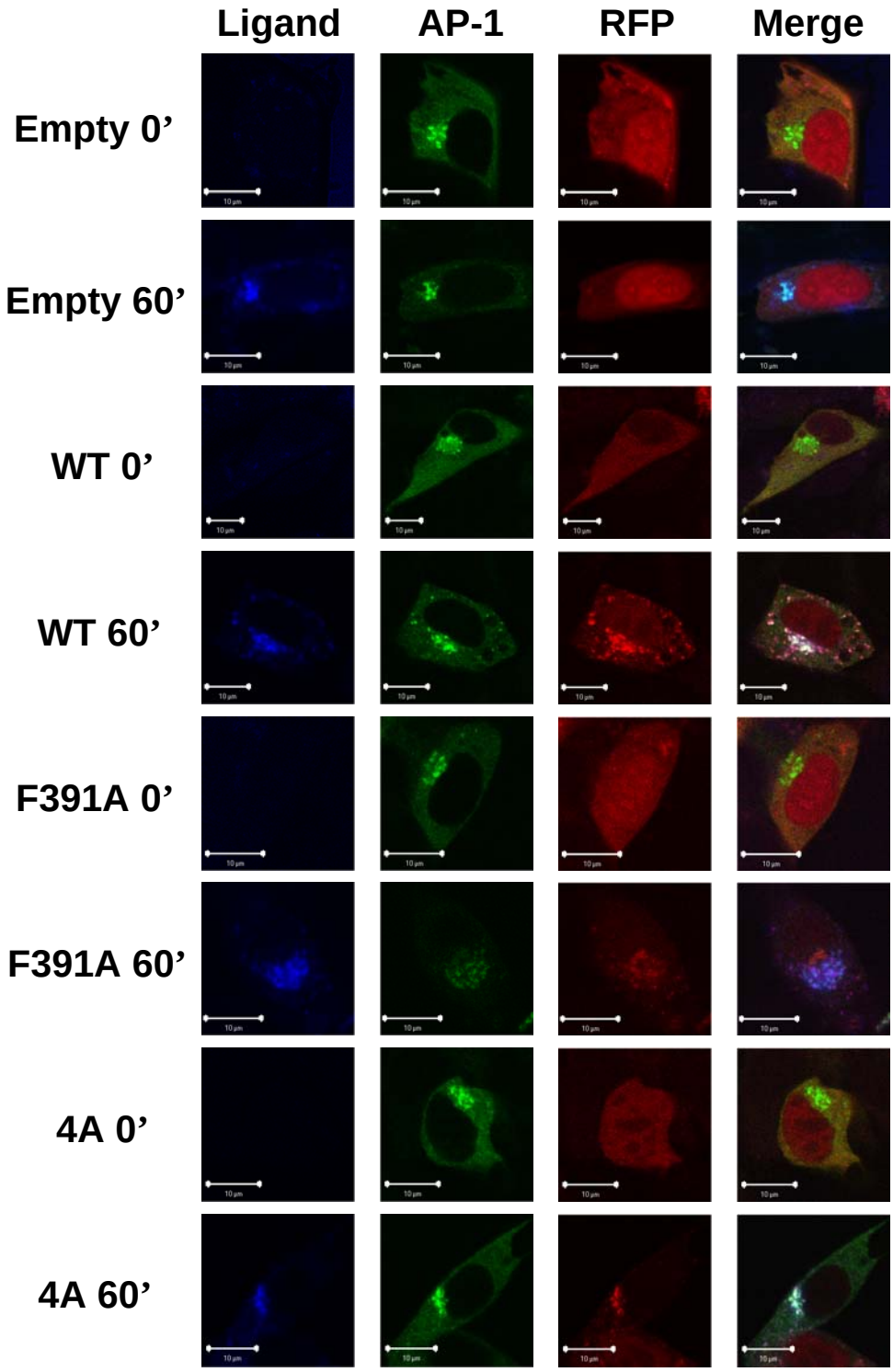

Figure 4. Arrestin mutants and trafficking with AP-1. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 63-3pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of three independent experiments.

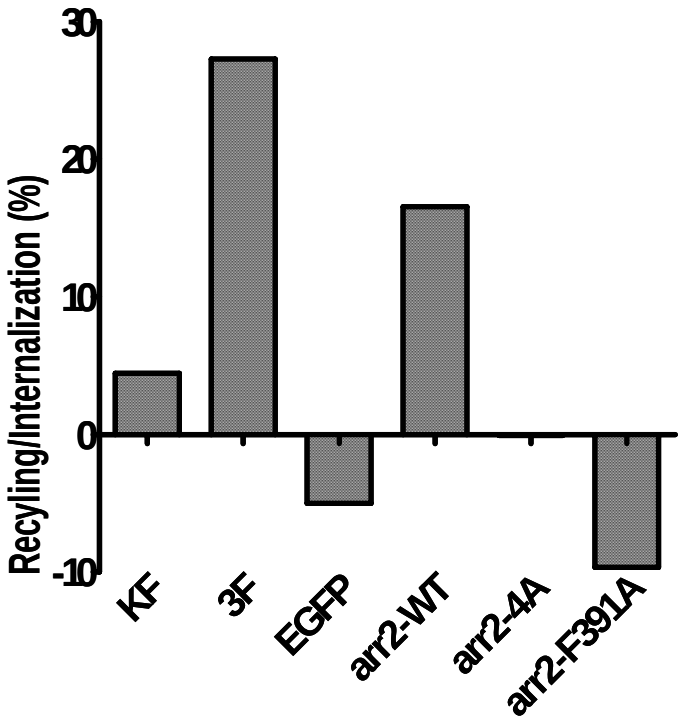

Figure 5. Recycling of the FPR in presence of arrestin-2 mutants. Transiently transfected KOFPRs were stimulated with 1µM fMLF, aliquoted before internalization, after stimulation and after recycling, washed 3 times with SFM and labeled with 633-6pep for analysis by flow cytometry. Data are expressed as mean and representative of one experiment.

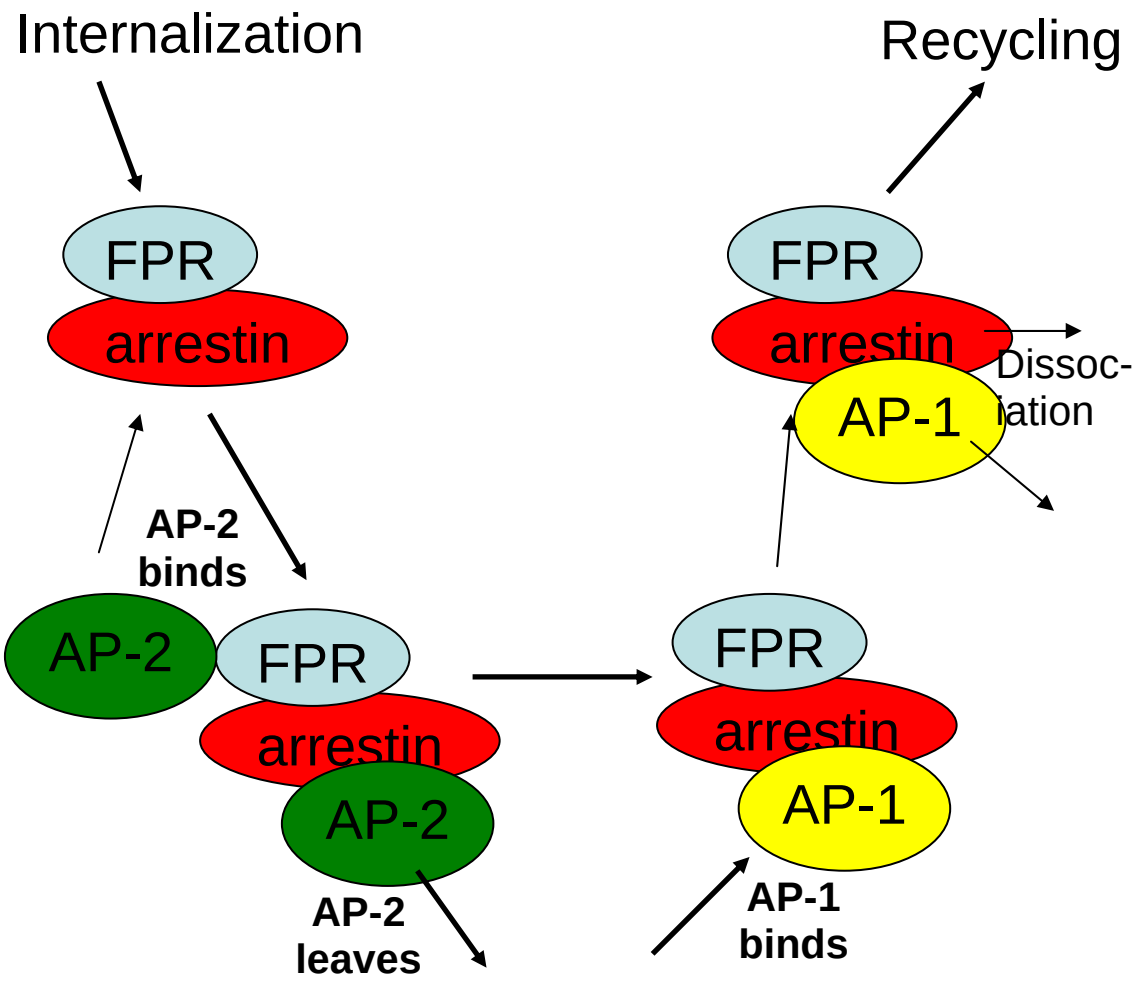

Figure 6. Model of FPR trafficking within the cell.

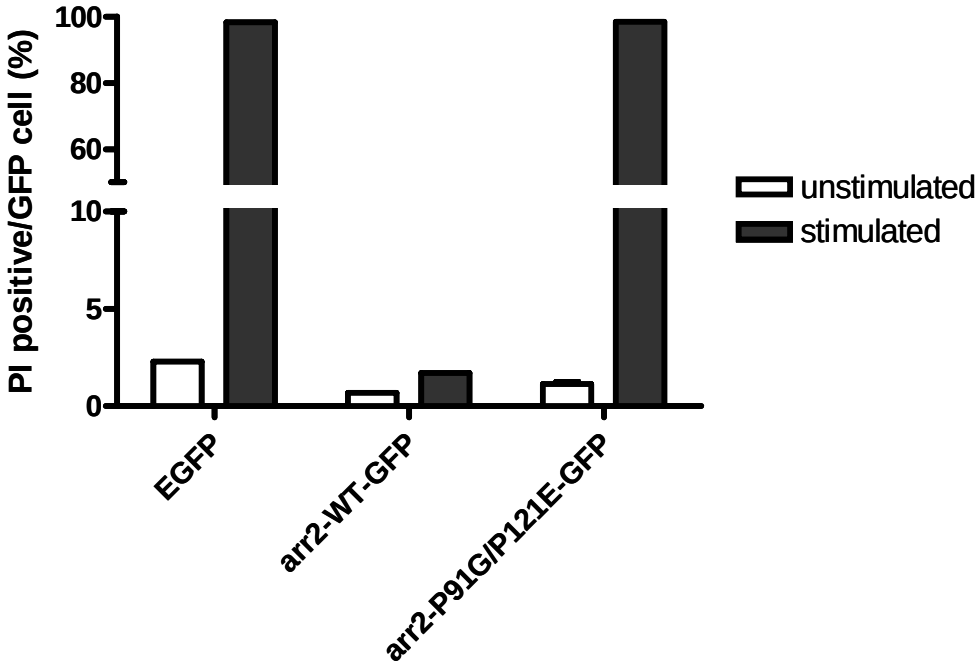

Figure 7. Src binding deficient arrestin mutants and their effect on FPR-mediated apoptosis. Transiently transfected KFs were plated on glass coverslips and stimulated with 10nM 633-6pep for five hours. Cells were stained with 100pg/µL PI, washed, fixed, mounted and assayed for percentage PI positive cells/GFP expressing cells. Data expressed as mean ±SEM and are representative of one experiment.

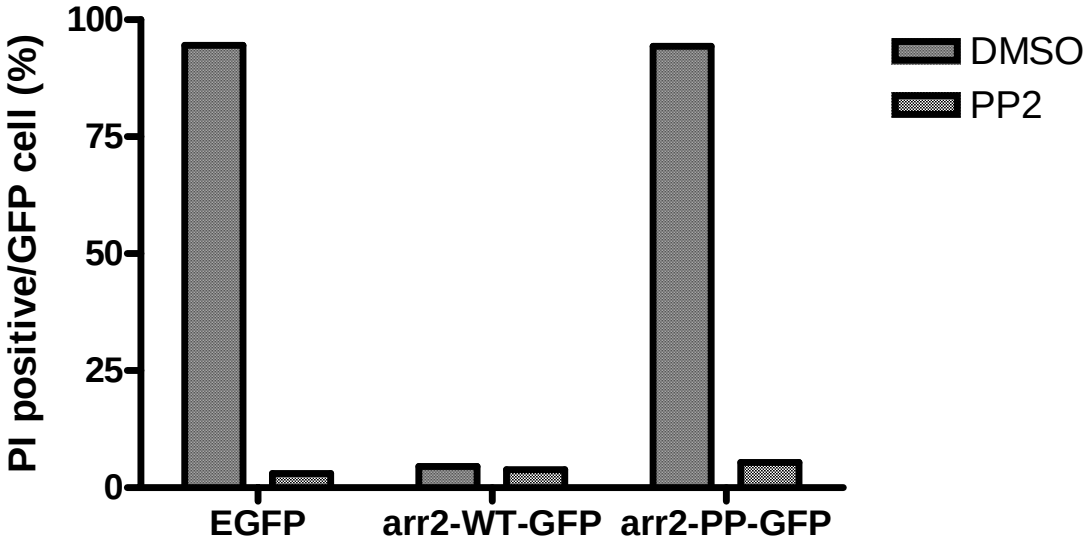

Figure 8. Src binding deficient arrestin mutants apoptosis is sensitive to PP2. Transiently transfected KFs were plated on glass coverslips, pretreated with 10nM PP2 and stimulated with 10nM 633-6pep for five hours. Cells were stained with 100pg/µL PI, washed, fixed, mounted and assayed for percentage PI positive cells/GFP expressing cells. Data expressed as mean and are representative of one experiment.

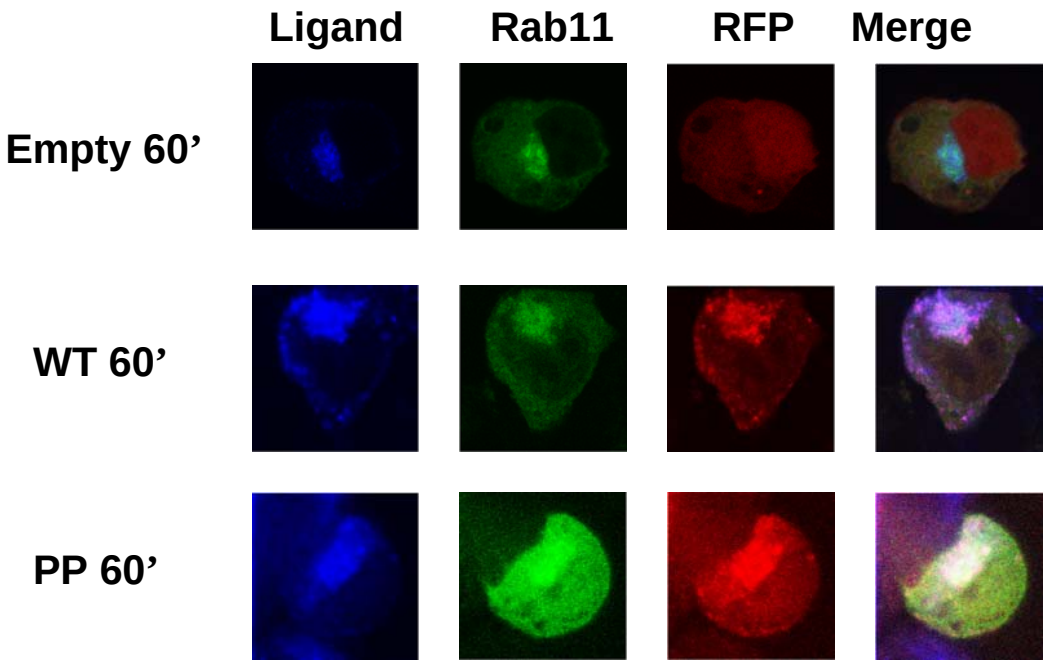

Figure 9. SH3-binding mutant trafficking with Rab11. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 633-6pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of three independent experiments.

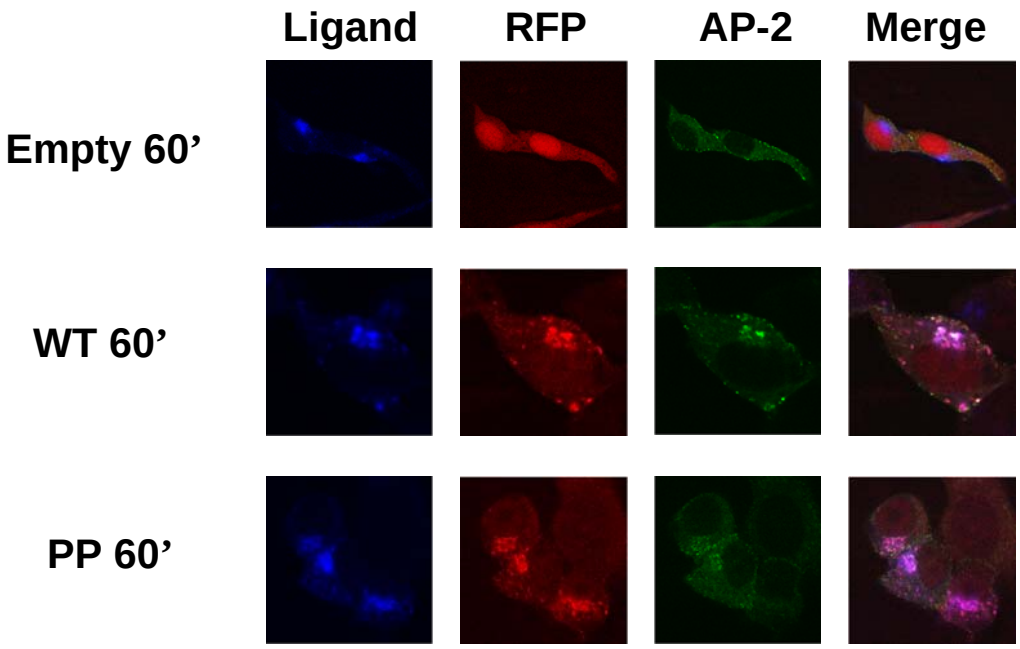

Figure 10. SH3-binding mutant trafficking with AP-2. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 633-6pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of three independent experiments.

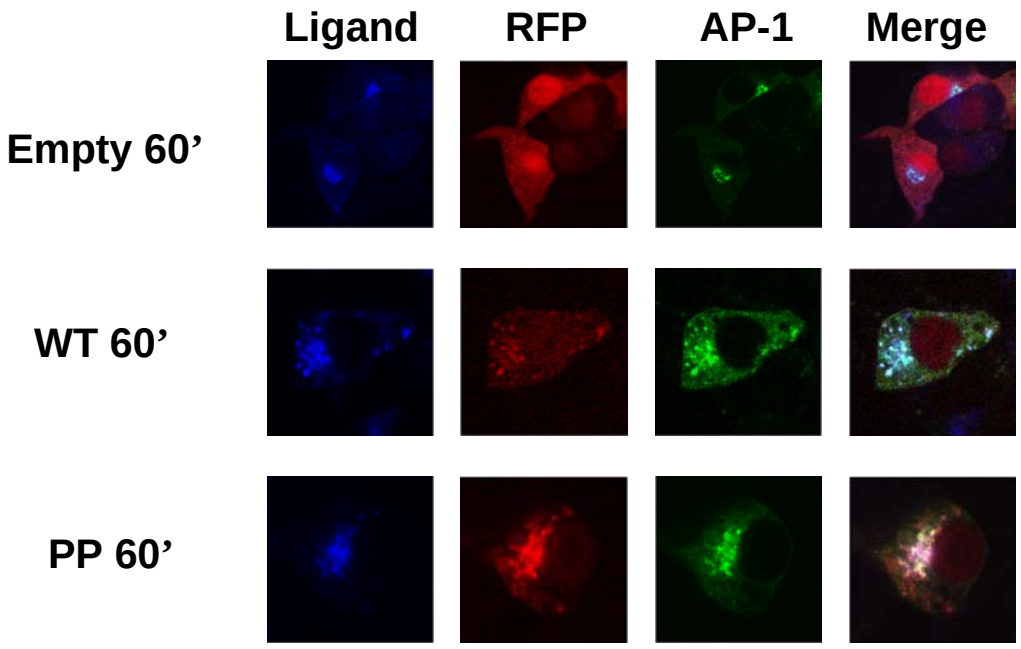

Figure 11. SH3-binding mutant trafficking with AP-1. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 633-6pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of three independent experiments.

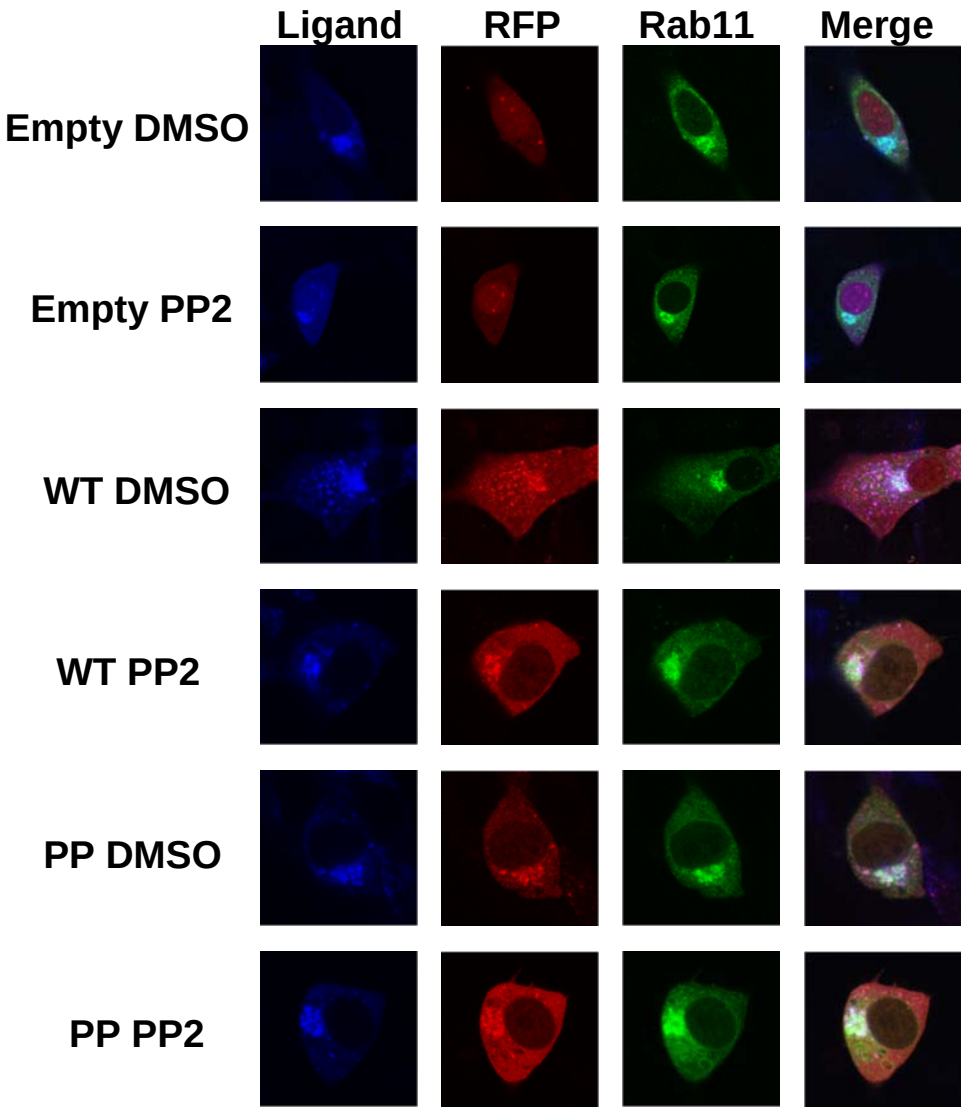

Figure 12. SH3 binding arrestin mutant sensitivity to PP2. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 633-6pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of one experiment.

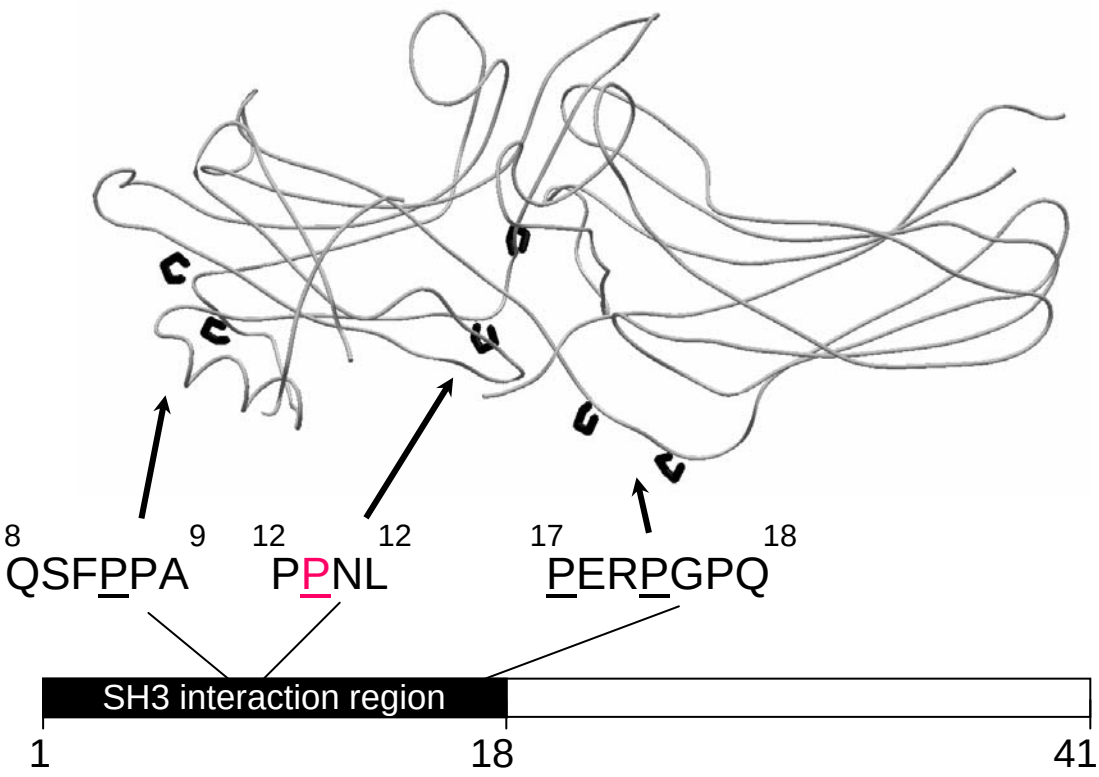

Figure 13. SH3-binding motifs in arrestin-2 that have been mutated to alanine.

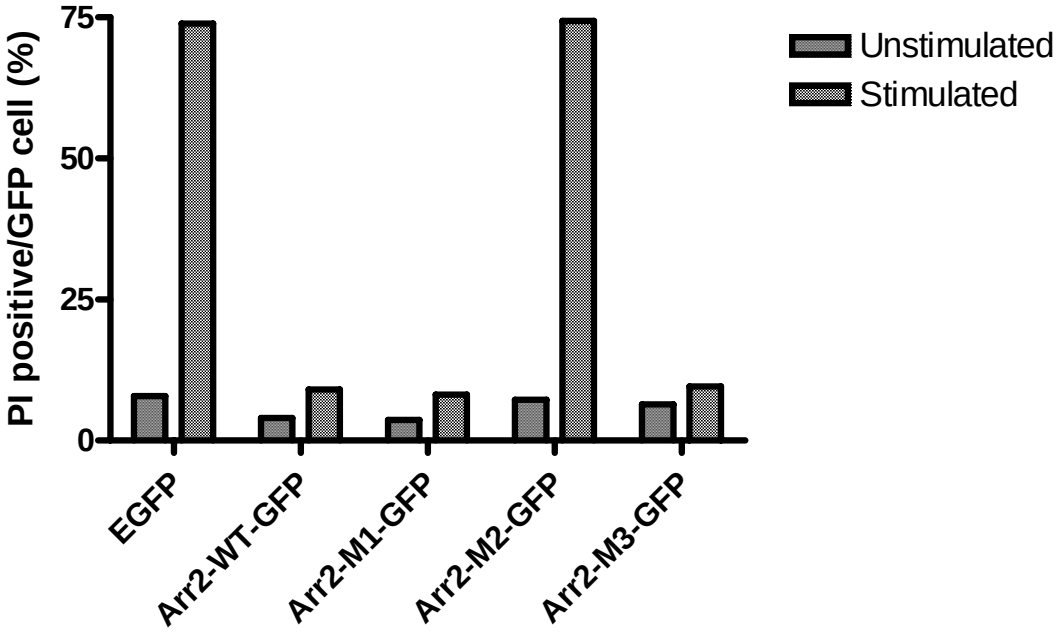

Figure 14. SH3-binding deficient arrestin mutants and their effect on FPR-mediated apoptosis. Transiently transfected KFs were plated on glass coverslips and stimulated with 10nM 633-6pep for five hours. Cells were stained with 100pg/µL PI, washed, fixed, mounted and assayed for percentage PI positive cells/GFP expressing cells. Data expressed as mean ±SEM and are representative of one experiment.

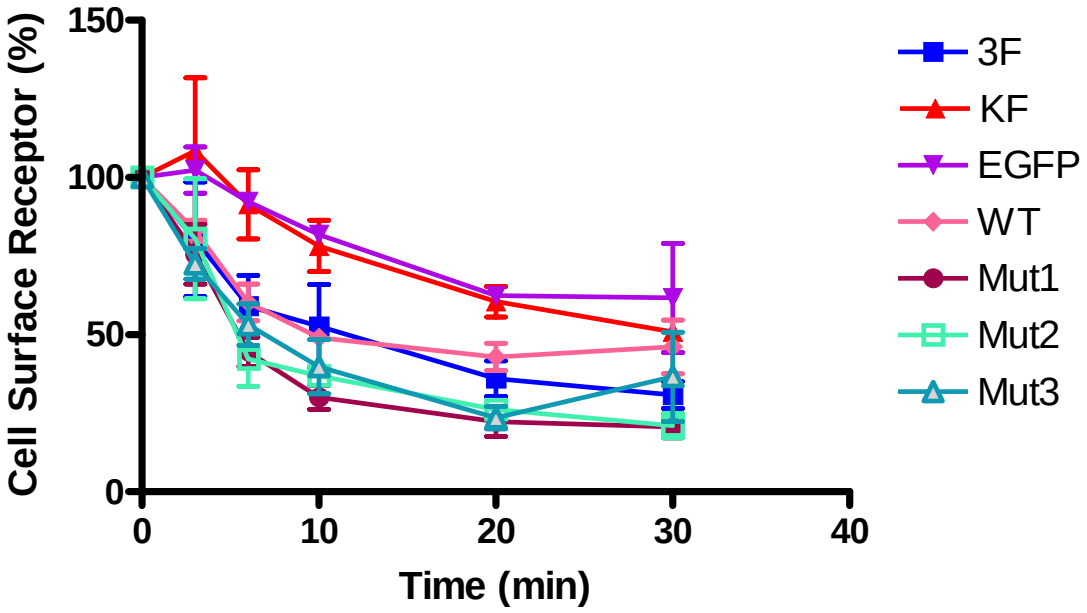

Figure 15. Internalization of the FPR in presence of arrestin-2 mutants. Transiently transfected KFs were stimulated with 1μM fMLF, aliquoted at time points shown, washed 3 times with SFM and labeled with 633-6pep for analysis by flow cytometry. Data are expressed as mean±SEM and are representative of three independent experiments.

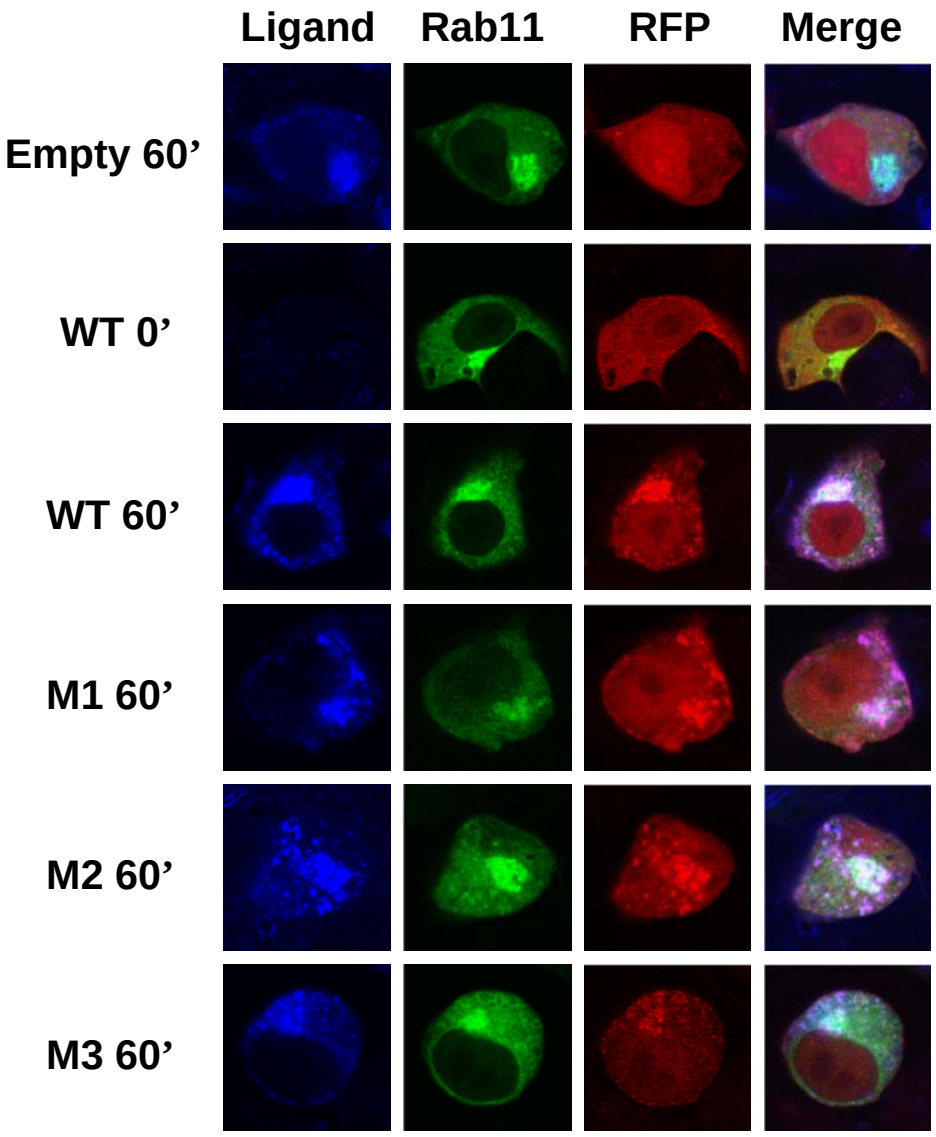

Figure 16. Src-binding mutant trafficking with Rab11. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 633-6pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of three independent experiments.

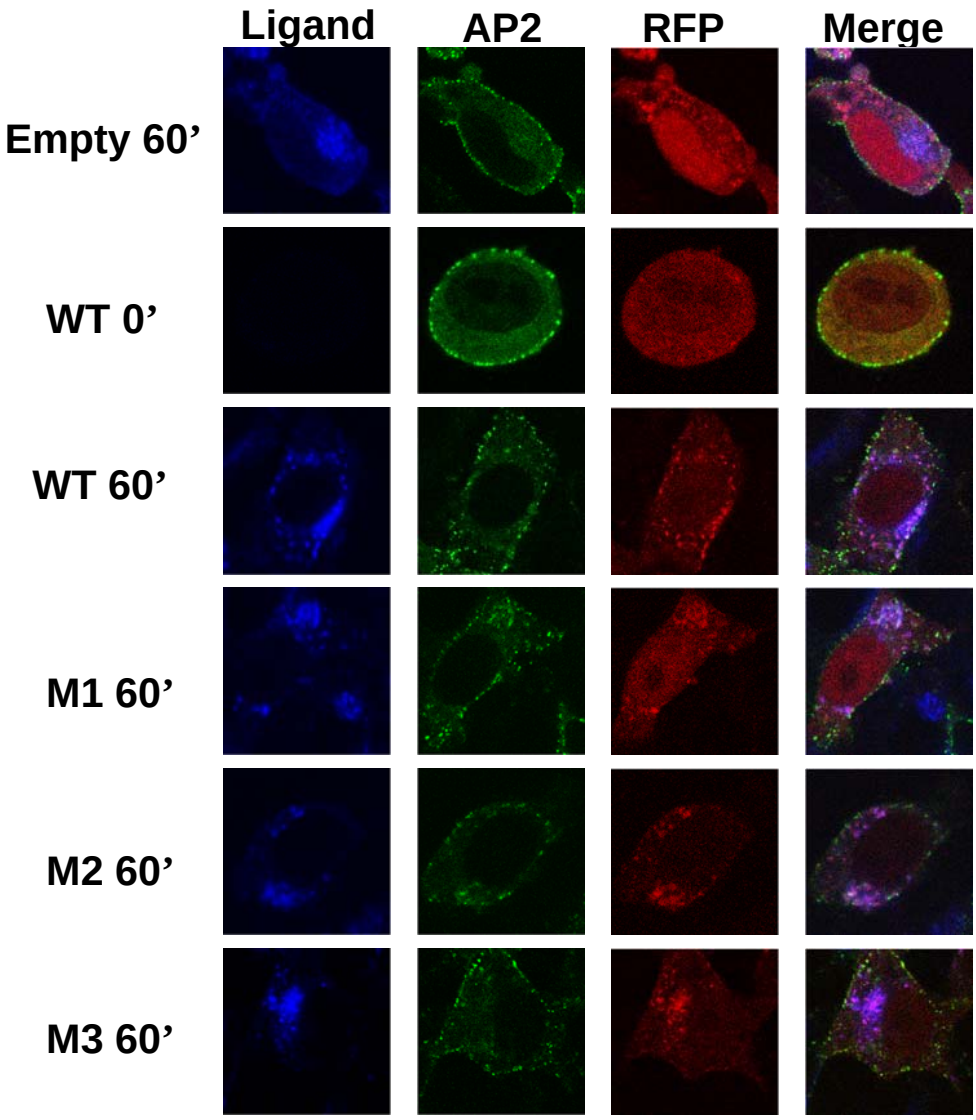

Figure 17. Src-binding mutant trafficking with AP-2. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 633-6pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of three independent experiments.

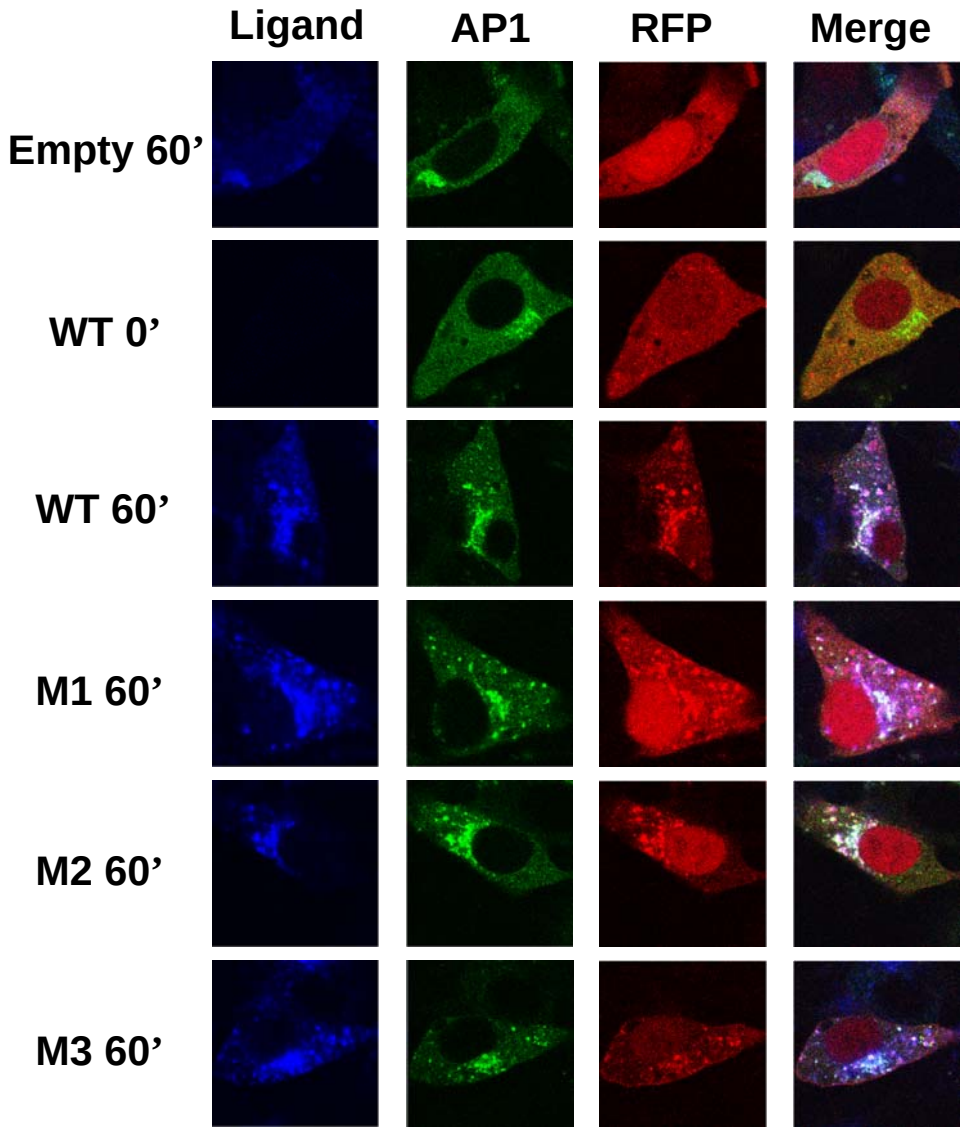

Figure 18. Src-binding mutants trafficking with AP-1. Transiently transfected KOFPRs were plated on glass coverslips, stimulated with 633-6pep, fixed, mounted and viewed by confocal fluorescence microscopy. Representative images are shown and are indicative of three independent experiments.